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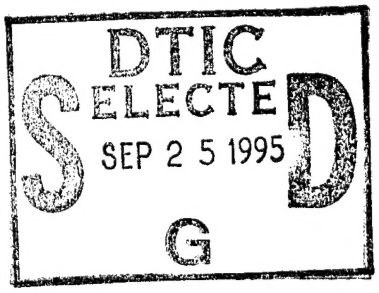

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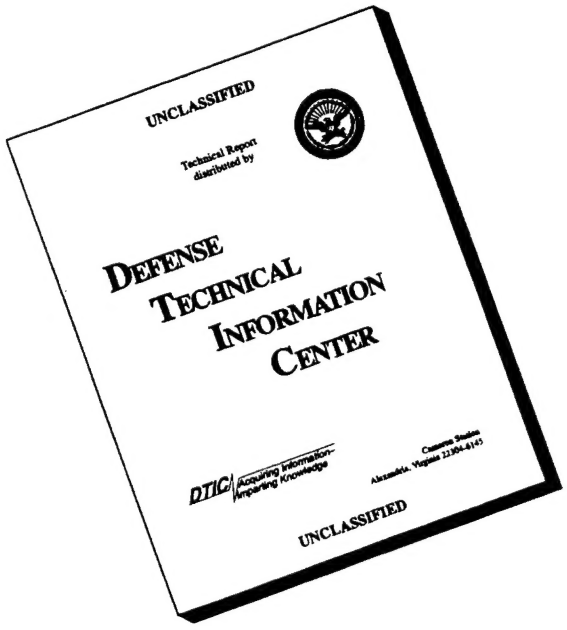

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We have identified serine 218 and serine 222 whose phosphorylations are critical for MEK activation both in vitro and in vivo. Interestingly, these two serine residues are differentially phosphorylated by Raf-1 and MEKK: Raf-1 phosphorylate serine 218 and serine 222 equally while MEKK preferentially phosphorylated serine 218.
In an attempt to study the potential of MEKK as an oncogene, we found that truncation of the putative N-terminal regulatory domain of MEKK failed to transform different cells. Instead stable overexpression of DMEKK appeared to be either lethal to growth inhibitory. To overcome the problem associated with constitutive overexpression of DMEKK, an inducible expression system was applyed Surprisingly, inducible expression of DMEKK had no effect on mapk activation in NIH3T3 cells whose MAPK pathways were still functional . Moreover we found that the recently discovered SAPK pathway was activated by DMEKK induction. Finally, in collaboration with other laboratories, we demonstrated that MEKK can directly phosphorylate and activate the SAPK activator SEK in vivo and in vitro. Therefore MEKK--SEK--SAPK defines a novel kinase cascade distinct from the Raf-1--MEK--MAPK kinase cascade.

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INTRODUCTION

Growth stimulation of cancer normal cells is accompanied by activateion of cytoplasmic kinase cascades. MAPKs(Mitogen-activated protein kinases) pathway is one of the best characterized signaling cascade which encompasses Raf-1 kinase, the MAPK activator MEK, and MAPKs. A good body of evidences supports a linear arrangement of this cascade: Raf-1 phosphorylates and activates MEK, which in turn phosphorylates and activates MAPK[1]. The MAP Kinase cascade seems to be highly conserved through revolution since several similar signaling cascades have been identified in the yeast systems[2]. On the basis of sequence homology to yeast kinases Byr2 and STE11, Lange-Carter et al. cloned another mammalian kinase capable to activate MEK *in vivo* when overexpressed in COS cells and to phosphorylate and activate MEK *in vitro*, hence named MEK Kinase (MEKK)[3].The proposed model is: MEK serves as a convergent point for signals from Raf-1 and MEKK which may transmit signals from different upstream components. In the kinase cascade from the activation of raf-1 to the activation of MAP kinase, only the mechanism by which MAP kinase is activated has been analysed in detail[4]. Little is known about the molecular detail of MEK activation other than that it is activated by phosphorylation, probably on serine residue(s). Moreover, it is interesting and important to clarify whether MEKK and Raf phosphorylate and activate MEK in the same or distinct way.

Protein Serine/Threonine kinase Raf-1 can be activated by a variety of mitogenic signals, including epidermal growth factor, platelet-derived growth factor, erythropoeitin, insulin, nerve growth factor and phorbol ester[5]. The essential role of Raf-1 in transduction of proliferative signals is demonstrated by the ability of antisense c-raf-1 RNA or dominant negative Raf-1 mutants to block the DNA synthesis and cell growth stimulated by serum and phorbol ester TPA[6]. Raf-1 has been positioned downstream of Ras in numerous signal transduction pathways and several lines of evidences have shown that Raf-1 might serve as one of the immediate targets of Ras[6, 7, 8, 9, 10, 11]. Regarding the consequences of raf-1 activation, both in vitro and in vivo data demonstrated Raf-1 activates MEK and MAPK.

Compared to Raf-1, there is relatively little information about the function and regulation of MEKK *in vivo*. In PC12 cells, it has been shown that the MEK phosphorylation activity of MEKK is rapidly activated by the treatment of EGF, NGF and TPA[12].Even though both overexpressed MEKK and v-Raf can activate MEK in COS cells and phosphorylate MEK *in vitro* ,unlike Raf-1, neither full length nor N-terminal truncated MEKK displays transforming activity in fibroblasts (such as NIH3T3 and Rat-1 cells, observation in Dr.Templeton's lab). In addition, the stable expression of N-terminal deletion mutant of MEKK (ΔMEKK) is either deleterious or growth inhibitory to the cells (unpulished data from Templeton's lab). This raises the question whether Raf-1 and MEKK regulate precisely the same downstream targets.

The existence of several functionally distinct kinase cascades in yeast and the structual conservation of these cascades, together with the fact that mammalian cells need differential responses to different environmental changes, suggests

that mammalian cell might employ kinase cascades other than MAP kinase cascade to response to extracellular alterations. The recently cloned and characterized Stress activated protein kinase (Sapk)/c-Jun N-terminal kinase (JNK) defines one of such kinase cascades[13, 14]. Even though there is overlapping in the activation of Sapk and Mapk by different stimuli, cellular stress such as heat shock, translational inhibition, UV irradiation and inflammatory cytokines such as TNF-a and IL-1 more efficiently activate Sapk than mitogens which, in contrast, more strongly activate MAPK[13]. Both ras-dependent and ras-independent pathways seem to be involved in the activation of Sapk[14, 15]. However, the mechanism by which Sapk is activated is not clear: what are the upstream kinases that activate Sapk pathway?

Results:

1. Identification of two serine residues which are differentially phosphorylated by Raf-1 and MEKK(*see appendex A*[16]).

To identify phosphorylation sites on MEK-1, we relied on a T7 promoter based mammalian cell overexpression system[17, 18] that has been very well adapted and extensively used in our laboratory. The vector pTM1 we used has been modified to encode one of two different peptide epitopes that are placed in the amino-terminal of expressed kinases. One epitope tag "EE" (EEEEYMPME) is derived from middle T antigen of polyomavirus and the other one "HA" epitope (YPYDVPDYA) from influenza virus.

Expression plasmids:

cDNA of Rat MEK-1 was cloned by reverse transcriptase-polymerase chain reaction according to published sequence and then subcloned into 5'EE pTM1. ΔRaf is a truncated form of human Raf-1(obtained from ATCC), in which the N-terminal 303 amino acids are deleted. A cDNA of p42 Mapk was obtained from Dr. Micheal Weber and also cloned into the 5'EEpTM1 or 5'HApTM1. ΔMEKK, an N-terminal 367 deletion form of MEKK1 was cloned by Louis Parrott in our Lab using RT-PCR and expressed as a 5'EE or 5'HA tagged protein using pTM1 as a vector. In addition, "KR" mutants for each of the kinases were generated by substituting the conserved Lysine residue in the kinase domain with Arginine: for ΔRaf, K375R; for ΔMEKK, K447R; for MEK-1, K97R; and for MAPK , K46R. A PCR based site-directed mutagenesis method was used to make all these mutants.

Reconstitution of MAPK activation in T7 overexpression system

In vaccinia T7 overexpression system, when MAPK was singly expressed, it appeared as a single band which represents unphosphorylated and

enzymatically inactive form of MAPK (as confirm by *in vivo* phospho labeling experiment and *in vitro* kinase assay). Coexpression with MAPK activator MEK-1 did not change this pattern. However, coexpression with either ΔRaf or ΔMEKK resulted in the appearance of a slow migrating band of MAPK which corresponded to the phosphorylated and kinase active form of MAPK. Inclusion of MEK-1 in the transfection almost completely converted MAPK into the upper band . Therefore activation of MAPK can be reconstituted in this system.

In vitro phosphorylation of MEK-1 by ΔRaf and ΔMEKK.

In order to identify the phosphorylation site(s) on MEK-1, the first piece of information needed to know is which type of amino acid is phosphorylated. To do this, EE tagged MEK-1 KR (as substrate) and ΔRaf and ΔMEKK were expressed in CV1 cells individually and purified by immunoprecipitation followed by elution with EE peptide. The eluted proteins were then used for *in vitro* kinase assays. The phosphoproteins were separated on SDS-PAGE, electrotransfered to Immobilon and visualized by autoradiography. Both purified ΔRaf and ΔMEKK can strongly phosphorylate MEK-1 KR. The phosphoamino acid analysis indicated that both ΔRaf and ΔMEKK phosphorylate MEK-1 only on serine residues.

Serine 218 is one of the phosphorylation site

If activation of MEK is conserved through its evolution, we speculate that the significant serine residues should either be identical in other alleles or possibly altered to threonine residues. Based on this, some of the potential serine residues were individually mutated to alanines in the background of KR MEK-1 and similar *in vitro* kinase assay was performed. Of these mutants, S218A demonstrated significantly reduced phosphorylation level even though comparable amount of protein was used , suggesting serine 218 is a possible phosphorylation site. To confirm this, I took advantage of the fact that MEK-1 was only phosphorylated on serine residue(s) and mutated this codon to a threonine residue. The level of phosphorylation of this S218T mutant by ΔMEKK was equivalent to that of the wild type MEK-1. Significantly, about 80% of the radiophosphate was contributed by threonine residue with the remainder by serine residue. Therefore, S218 is one of the phosphorylation sites.

Identification of another phosphorylation site

One of chymotryptic phosphopeptide obtained from the *in vitro* labeled S218T MEK-1 contained both phosphoserine and phosphothreonine(data not shown), suggesting that at least another phosphoserine was localized in the same peptide as phosphothreonine 218 did. Serine 218 lies within a domain similar to one in MAPK phosphorylated on two clustered residues. This led us to speculate that MEK-1 might be doubly phosphorylated in a similar manner. Each of the two serines near Serine 218 (serine 212 and serine 222) was mutated to threonine and only S222T MEK-1 could be phosphorylated on threonine residue by ΔRaf or ΔMEKK, suggesting serine 222 was another phosphorylation site. Finally, the double mutants S218T/S222T was only phosphorylated on threonine while S218A/S222A was just barely phosphorylated. Therefore serine 218 and serine 222 are two phosphorylation sites by ΔRaf and ΔMEKK.

Raf and MEKK show different site preference for MEK phosphorylation.

The phosphorylation kinetics analysis of codon 218 and codon 222 using mutants MEK S218T and MEK S222T as substrates revealed that ΔMEKK phosphorylate codon 218 more rapidly than codon 222 irrespective of whether it was a serine or threonine, contrary to ΔRaf which phosphorylated these two codons nearly equally at all time points.

Phosphorylation of these two sites is important for MEK-1 activation in vitro.

In order to activate MEK-1 *in vitro*, ΔRaf and ΔMEKK immunopurified from transfected CV1 cells were used to phosphorylate wild type MEK-1 or double alanine-substituted MEK-1 in the presence of 50 uM cold ATP in kinase reaction buffer for 30 minutes. KR MAPK was then added together with γ-^{32}P-ATP and the kinase reactions were stopped after another 30 minutes incubation. Both ΔRaf and ΔMEKK were able to activate wild type MEK-1 in vitro, whereas S218A/S222A mutant was unable to be activated.

Phosphorylation of these two sites is important for MEK-1 activation in vivo.

To test whether phosphorylation of these two serine residues which are phosphorylated *in vitro* is important for MEK-1 activation *in vivo*, the effects of mutations of either one serine residue to a nonphosphorylatable alanine were evaluated. Wild type MEK-1, single-site MEK mutants or double-site mutant (all N-terminal "EE" tagged) were coexpressed with either wild type ΔRaf or KR ΔRaf (not tagged) in CV1 cells. The epitope tagged MEK were immunopurified by Affi-Gel beads coupled with anti-EE antibody and kinase activities were measured by using KR MAPK as a substrate. Both single-site mutations partially reduced the activities of MEK-1 while double-site mutation completely eliminated the kinase activity of MEK-1.

2. Activation of stress activated kinase pathway by MEKK (*see appendex B*[19])

As an initial step to study the function of MEKK *in vivo*, we have attempted to establish cell lines able to stably overexpress ΔMEKK. ΔMEKK was cloned into a

CMV promoter-based vector that has been modified to attach an N-terminal "EE" epitope tag to the open reading frame of gene of interest. In transient transfection experiments, the expression of "EE" ΔMEKK was easily detected by immunoblot using "EE" antibody. However, I have failed to obtain stable cell lines that expressed detectable amount of ΔMEKK from Hela cells, NIH3T3 cells, Rat-1 cells and CV1 cells. In parallel experiments, we successfully obtained cell lines that overexpressed MEK.2E (a constitutively active MEK mutant substituting two glutamic acids for serine residues that are phosphorylated during activation). Moreover, when ΔMEKK expression plasmid was cotransfected with drug resistance plasmid, the number of colonies after drug selection was much fewer compared with that of the control transfection. All these suggested that stable overexpression of ΔMEKK was lethal to or growth inhibitory on cells tested. To overcome the problem associated with constitutive overexpression, I turned to an inducible expression system to study the biological function of MEKK *in vivo*.

Establishment of NIH3T3 cell lines that inducibly expressed ΔMEKK.

The LacSwitch system (Strategen) was chosen to establish the inducible expression cell lines. The ΔMEKK including N-terminal "EE" epitope tag was cloned into the pRSV-IOP3 vector whose expression was detected by immunoprecipitation followed by anti-EE immunoblot.
NIH3T3 cells were transfected with p3'SS and pEE-ΔMEKK-IOP3 at a ratio of 3:1 using standard Calcium phosphate method. The cells were let to recover for one day before splitting into selection medium containing 500 ug/ml G418. Individual clones were picked and grown up. The inducibility of ΔMEKK expression of each G418 resistant clone was tested in the presence or absence of 1 mM IPTG. Of about 30 clones tested, one third of them turned out to be positive and two of them which displayed the lowest basal level and the highest inducibility of ΔMEKK expression were chosen for further studies.

Inducible expression of ΔMEKK by IPTG.

The expression of ΔMEKK could be clearly detected after 12 hours of IPTG induction. Significantly, the effect on Sapk activation was detectable only after 3 hours induction by IPTG(see below).

Cell growth inhibition associated with ΔMEKK expression

One apparent consequence of IPTG induction was the cell growth inhibition, a

common characteristics of all inducible clones, even though to various extent. Actually, these cells demonstrated slower cell growth than parental NIH3T3 cells even without IPTG, possible due to leaky expression of ΔMEKK within these cells. The mechanism of this observed cell growth inhibition is not clear.

Activation of SAPK but not MAPK by MEKK in vivo.

If MEKK is an activator of MEK as Raf, it is expected that expression of ΔMEKK would activate MAPK in these cells. To our surprise, induction of ΔMEKK expression in these two clones had no effect on MAPK activation. However, in these cells the MAPK pathways were still functional because treatment of TPA strongly activated MAPK. TPA did not activate SAPK in these cells, consistent with the previous report that in NIH3T3 cells SAPK was not activated by TPA treatment. Then we tested whether SAPK could be a downstream target of MEKK. Indeed, induction of IPTG activated SAPK in the inducible cells but not in the parental cells. The activation of SAPK was evident 3 hours after induction and reached maximum after 12 hours, while MAPK activities remained essentially unchanged throughout the 23 hour incubation with inducer. Taken together, these results suggested that MEKK can function to activate SAPK pathway which is separated from MAPK pathway.Therefore MEKK appears to have a distinct role in activating downstream signals from Raf-1. At least in the experimental system I have used, MEKK differentially activate Sapk pathway but not MAPK pathway. Furthermore, in collaboration with other laboratories, we have shown that MEKK can directly phosphorylate and activate the SAPK activator SEK in vivo and in vitro[20].

Conclusions:

In vitro and in vivo overexpression system, both Raf and MEKK can phosphorylate and activate MEK by phosphorylating two critical residues S218 and S222. Biochemically, however, Raf and MEKK are not identical because Raf shows no preference to these two sites while MEKK differentially phosphorylate one of them. In the inducible expression system, the relatively low level expression of ΔMEKK specifically activated the SAPK pathway but not MAPK pathway which was preferentially activated by TPA treatment. Appreciation of these two distinct signaling pathways will help better understand the growth regulation of both cancer and normal cells in response to diverse external signals.

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Appendex A Yan,m

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Identification of 2 Serine Residues of MEK-1 That Are Differentially Phosphorylated during Activation by *raf* and MEK Kinase*

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The signal transduction kinase MEK (mitogen-activated protein (MAP) or extracellular signal-regulated (Erk) kinase)-1 is activated via phosphorylation by MEKK (MEK kinase) and *raf* kinases. We show here that these two kinases phosphorylate rat MEK-1 exclusively on two serine codons, Ser^{218} and Ser^{222}. Phosphorylation of MEK-1 on serines 218 and 222 is both necessary and sufficient for MEK-1 to be activated and able to phosphorylate MAP kinase. A mutant form of MEK-1 that replaces these two codons with alanine cannot be activated, and one that substitutes glutamic acid residues in place of these 2 serines is active independent of activation by phosphorylation. These sites of activation occur in a region of MEK-1 that is similar to sites of activating phosphorylation in several other serine/threonine kinases, suggesting that this region may represent a conserved "activating domain" of many kinases. MEKK and *raf* display differences in site preference between these two codons, with MEKK showing preference for the amino acid at codon 218 and *raf* phosphorylating each residue approximately equally. This site preference might result in differences in the temporal or subsequent substrate patterns of MEK activation that result from these two activation pathways.

Cell growth signal transduction is frequently accompanied by phosphorylation and activation of MAP[1] kinase, as reviewed recently (1). Two kinases capable of activating MAP kinase have been cloned, one termed MEK-1 (MAP or Erk kinase; alternatively MAP kinase kinase-1) (2, 3) and the other MAP kinase kinase-2 (4). MEK-1 is in turn activated by phosphorylation by the v-*raf* oncoprotein (5). Activation of c-*raf* is stimulated by the *ras* activation pathway, and recently direct interaction of c-*raf* with the *ras* oncoprotein has been demonstrated (6–9).

A kinase structurally unrelated to *raf* is similarly able to phosphorylate and activate MEK-1. This kinase, termed MEK kinase (or MEKK) because it lies immediately upstream of MEK, has recently been cloned (10), and several groups have characterized the enzymatic activity (see Ref. 11 for review). Although the growth signal that passes through MEKK might originate separately from the signal that passes through *raf*, the net effect, *i.e.* activation of MAP kinase, seems equivalent (10).

Since MEK-1 lies at the convergence of signaling pathways that may have distinct origins, the molecular details of MEK regulation are important. We have investigated the nature of MEK-1 activation by *raf* and MEKK and have developed *in vivo* and *in vitro* systems to model the activation of MEK. Using these model systems, we have identified the amino acid residues upon which *raf* and MEKK phosphorylate MEK-1 and show here that these sites are necessary for activation of MEK-1 *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture and Gene Expression—Kinases were expressed in CV1 monkey kidney cells (ATCC) maintained in Dulbecco's modified Eagle's medium with 10% calf serum using the plasmid vector pTM1 (12, 13), which encodes protein products under the control of the bacteriophage T7 promoter. To express the proteins in eukaryotic cells, the plasmid-transfected cells were infected with the recombinant vaccinia virus vector vTF7-3 that encodes the T7 RNA polymerase and then were transfected with the plasmid vector. Transfection and infection of CV1 cells using this system has been described previously (14).

Plasmid Constructions—Rat MEK-1 (2) and mouse MEKK (10) were cloned by reverse transcriptase-polymerase chain reaction using primers designed from the published sequence. The coding regions were expressed as an epitope-tagged protein, with a synthetic amino-terminal epitope (EEEEYMPME, termed "EE") derived from middle T antigen of polyomavirus. This antibody, and suggestions for its use, were from Gernot Walter, University of California, San Diego. In this paper, we have used exclusively a truncated active fragment of MEKK, representing a deletion of the amino-terminal 367 amino acids, termed ΔMEKK. A truncated active form of c-*raf*, in which the 303 amino-terminal amino acids are deleted, was also used in these experiments (termed Δ*raf*) and was also epitope tagged with the EE epitope. A cDNA encoding murine MAP kinase (p42) was obtained from Michael Weber (University of Virginia) and was modified by addition of an amino-terminal EE epitope as for the other kinases. Inactive "KR" mutations used were, for Δ*raf*, K375R; for ΔMEKK, K447R; for MEK-1, K97R; and for MAPK, K46R.

Mutagenesis—A variation of the "megaprimer" PCR method of mutagenesis (15) was used. Small restriction fragments containing the mutagenized region were subcloned into unmutagenized plasmids and the entire mutagenized region sequenced to assure against unexpected mutations.

Immunopurification of Kinases—Transfected cells expressing epitope tagged kinases were lysed in MLB (50 mM MOPS·Na, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, and 1 mM dithiothreitol, containing the protease inhibitors aprotinin (2.5 μg/ml), leupeptin (2.5 μg/ml), and phenylmethylsulfonyl fluoride (50 μg/ml) and the phosphatase inhibitors NaF (10 mM), sodium pyrophosphate (5 mM), Na_3VO_4 (1 mM), and β-glycerol phosphate (10 mM). After clarification of the cell extracts, epitope-tagged kinases were precipitated using Affi-Gel 10 beads (Bio-Rad) coupled to purified EE monoclonal antibody (3 mg of antibody/ml of gel). To purify protein from 10^7 transfected cells, 20 μl of this affinity matrix was used. Immune complexes were collected and washed in MLB by spin filtration using microfiltration columns ("Compact Reaction Columns," U. S. Biochemical Corp.). Bound proteins were eluted overnight in 25 μl of elution buffer (50 mM Tris·Cl, pH 7.4, 0.5 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 20% glycerol), including 40 μg/ml EE peptide (*N*-Ac-EEEEYMPME-COOH), and the filtrate recovered by centrifugation. Typically, the resulting solution contained 50–200 ng of kinase/μl, and the immunopurified kinase is the only protein detected using Coomassie gel staining.

In Vitro Phosphorylation—Routinely, 1 μl of the recovered kinase from the procedure above was used to phosphorylate 4 μl of an identically purified kinase substrate. The 20-μl reaction mixture contained 10

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[1] The abbreviations used are: MAP, mitogen-associated protein; MEK, MAP or extracellular signal-regulated (Erk) kinase; MEKK, MEK kinase; MAPK, MAP kinase; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

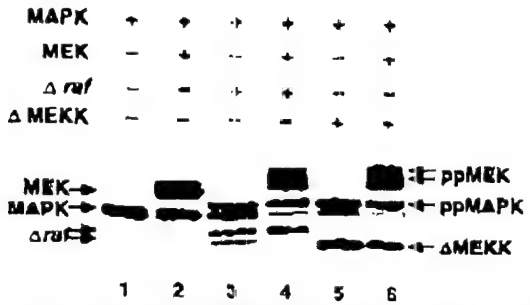

FIG. 1. ***In vivo* modeling of MAPK and MEK-1 activation.** MAPK was expressed in CV1 cells together with MEK-1 with or without activated forms of MEKK or *raf* as indicated. Each of the kinases were tagged with an amino-terminal synthetic epitope ("EE"), and the expression of each protein was monitored using anti-EE antibody for immunoblotting. MAPK expressed alone (*lane 1*) is minimally phosphorylated, as evidenced by the absence of the slowly migrating phosphorylated form. Co-expression of MEK with MAPK (*lane 2*) is insufficient to increase MAPK phosphorylation, unless an activating kinase such as *raf* (*lanes 3* and *4*) or MEKK (*lanes 5* and *6*) is included. In this case, MEK displays several forms with reduced mobility consistent with phosphorylation. MAPK is phosphorylated in response to the *raf* or MEKK activators, even more so in conjunction with MEK-1. Additionally, Δ*raf* shows a slowly migrating phosphorylated band, especially when expressed together with MEK (*lane 4*).

FIG. 2. **Phosphorylation of MEK-1 *in vitro*.** ΔMEKK and Δ*raf* were immunopurified as described under "Experimental Procedures" and used to phosphorylate a kinase inactive form of MEK-1, termed MEK-KR. MEK-KR is strongly phosphorylated by both MEKK and *raf*, but is devoid of autophosphorylating activity, since it is not phosphorylated when incubated without the other kinases (*lane 3*). ΔMEKK is also strongly phosphorylated, and Δ*raf* is slightly phosphorylated, faintly visible in this experiment, migrating between ΔMEKK and MEK.

μM ATP, 10 μCi of [γ-^{32}P]ATP, in 50 mM Tris·Cl, pH 7.4, 0.5 mM dithiothreitol, 10 mM $MgCl_2$. After 30 min at room temperature, reaction was stopped by the addition of SDS/dithiothreitol sample buffer and boiling. Because we found that the epitope-tagged Δ*raf* kinase was only fractionally eluted from the beads using this procedure, in some reactions (those in Fig. 7) reactions were performed using kinases that remained attached to the immune affinity beads. Kinase reaction conditions were otherwise as shown above.

Phosphoamino Acid Analysis—Phosphoproteins blotted onto Immobilon were hydrolyzed in 6 M HCl at 110 °C for 1 h according to Kamps (16) and analyzed by thin layer electrophoresis at pH 3.5.

RESULTS

Expression of Signal Transduction Kinases—We used the T7-polymerase gene expression system of Moss (12, 13) to express MAP kinase (p42), MEK-1, and truncated kinase-active versions of MEKK and *raf*, either alone or in combinations within cells. To monitor expression of these protein kinases, and to afford simple means of micropurification of the wild type or mutant forms, we modified each of the proteins by the addition of a 10-amino acid synthetic epitope to the amino terminus of each protein. Each of the kinases is thus identifiable by Western blotting against the synthetic epitope (termed EE) and can be immunopurified under nondenaturing conditions.

When expressed alone using this system, MAPK is almost completely unphosphorylated (as shown in Fig. 1, *lane 1*), and phosphorylation of MAPK is unaffected by co-expression of MEK-1 (*lane 2*). This is probably because MEK itself is dependent upon upstream activation. When MAPK is expressed together with either Δ*raf* or ΔMEKK, increased phosphorylation of MAPK is seen, evidenced by a band with reduced electrophoretic mobility. Inclusion of MEK with these kinases results in nearly complete phosphorylation of MAPK (*lanes 4* and *6*), apparently due to expression of a complete activation pathway. In these lanes, MEK also shows a reduced electrophoretic migration compared with when it is expressed alone. This change reflects phosphorylation of MEK on serine and threonine residues (17) possibly from phosphorylation by Δ*raf* or ΔMEKK or by retrograde phosphorylation of MEK by MAPK or other kinases.

In Vitro Phosphorylation of MEK-1 by MEKK and raf—Activation of MEK-1 was also modeled *in vitro* using the active forms of MEKK or *raf* to phosphorylate MEK-1. To do this, we immunopurified epitope-tagged forms of both of these kinases (see "Experimental Procedures") and similarly purified a kinase inactive mutant of MEK-1 (containing a lysine to arginine mutation within the ATP binding domain of the kinase (18)). An example of this is shown in Fig. 2, in which both ΔMEKK and Δ*raf* are used to phosphorylate MEK-KR protein (*lanes 1* and *2*), whereas the preparation of substrate MEK-KR is itself completely devoid of phosphorylating activity (*lane 3*). ΔMEKK is also strongly phosphorylated, apparently through an autocatalytic reaction, whereas Δ*raf* is weakly phosphorylated (*lane 2*, but more clearly seen in Fig. 4). In experiments not shown, we found that MAPK phosphorylated MEK-1 on serine and threonine residues, whereas MEKK and *raf* phosphorylated exclusively serine residues. Since *raf* and MEKK are able to phosphorylate MEK-1 only on serine residues, and this phosphorylation was sufficient to activate MEK kinase activity (see Figs. 4 and 5 below), we focused on serine residues as potential sites of activating phosphorylation.

Identifying the Sites of MEK-1 Phosphorylation by raf and MEKK—Inspection of the predicted amino acid sequence of the rat MEK-1 protein reveals 18 serine residues, of which some are not conserved in MEK alleles or the homologous counterparts of MEK in other species. If activation of MEK-1 is conserved through its evolution, we predicted that the significant serine residues should either be identical in other alleles or possibly could be altered to threonine residues. Based on these suppositions, we initiated a program of directed mutagenesis targeting several serine codons, preparing these mutants in a background of the inactivated K96R MEK mutant to eliminate autophosphorylation by MEK.

Phosphorylation of mutant MEK proteins using ΔMEKK or Δ*raf* kinases showed that mutant S218A demonstrated reduced phosphorylation despite equivalent expression levels (not shown). However, mutation of this residue might have caused unpredictable changes in protein conformation, resulting in decreased phosphorylation at a distant site. To confirm phosphorylation of serine 218 genetically, we mutated this codon to a threonine residue. The level of phosphorylation of mutant protein S218T by ΔMEKK was equivalent to that of the wild

type protein. However, about 80% of the radiophosphate in this mutant protein was localized to threonine residues with the remainder on serine residues. Because a small amount of phosphoserine was still detected in mutant S218T, we surmised that at least one more site of serine phosphorylation was present on MEK.

We identified the additional phosphorylation site based on two observations. First, one of two chymotryptic peptides obtained during peptide mapping of the *in vitro* labeled MEK S218T mutant contained both phosphoserine and phosphothreonine (data not shown). This suggested that the two phosphorylation sites were contained within the same chymotryptic peptide. Second, the Ser^{218} residue lies within a domain similar to one in MAP kinase phosphorylated on two clustered residues (see Fig. 6). This lead us to speculate that MEK might be doubly phosphorylated in a similar cluster. We subsequently mutated each of the two serine residues near Ser^{218}, creating threonine codons, and found that only one of these mutant proteins (S222T) was phosphorylated on threonine. A double mutant in which both serine codons at 218 and 222 are altered to threonine contained no phosphoserine when phosphorylated by MEKK or *raf*. These experiments are shown in Fig. 3.

These data show that activated *raf* and MEKK phosphorylate the same 2 residues on MEK-1. MEKK shows apparent preference for the Ser^{218} residue *in vitro*, whereas *raf* phosphorylates each site roughly equivalently, based on the levels of phosphorylation of the individual threonine mutants. This observation is examined more closely below in Fig. 7. Additionally, the data in Fig. 3 show that no sites other than the serines at 218 and 222 are phosphorylated, since the 218T/222T mutant displays no phosphoserine, and the wild type protein displays no phosphorylation on threonine or tyrosine.

Do the Identified Sites Confer "Activatability" to MEK-1 in Vivo?—If Ser^{218} and Ser^{222} are required to activate MEK-1, an important prediction to test is that mutation of these sites would eliminate the ability of MEK to be activated by *raf* or MEKK *in vivo*. To test this prediction, we expressed epitope tagged MEK-1 proteins (wild type or alanine substitutions at the 218 and 222 sites, all EE-tagged) with or without active *Δraf* proteins (without the EE epitope tag in this experiment). We then specifically immunopurified the epitope-tagged MEK proteins and assayed them for MAP kinase phosphorylation activity *in vitro*. As shown in Fig. 4, both of the single-site mutant proteins are still partly activated. Only the double alanine mutant demonstrated no activity either in the presence or absence of *raf*. Thus, both Ser^{218} and Ser^{222} contribute to the activatability of MEK by *raf*. The wild type MEK-1 protein was activated by co-expression with *Δraf*, but clearly displays some basal activity even in the absence of co-transfected active *raf*. Since the plasmid-encoded proteins are expressed in the full context of normal cellular proteins, this low level of MEK activation almost certainly results from activation by uncharacterized cellular kinase(s), probably including but not exclusively the endogenous *raf* and MEKK kinases.

Do the Sites Confer Activatability in Vitro?—Since the activation of MEK-1 by *raf* in Fig. 4 occurred in intact cells, it remained possible that activation of MEK was not a result of *raf*, but rather by an intermediate *raf*-activated kinase. To model the activation of MEK *in vitro*, we purified *raf* and MEKK and used these kinases to phosphorylate MEK or the alanine-substituted mutant, in the presence of unlabeled ATP. Phosphorylated MEK protein was then incubated with an inactive mutant (K46R) of MAPK to quantify MEK activity. As shown in Fig. 5, both *raf* and MEKK are able to activate wild type MEK-1 *in vitro*, whereas the mutant substituting alanine residues at codons 218 and 222 is unable to be activated. Thus, these codons are required for direct activation by either *raf* or MEKK.

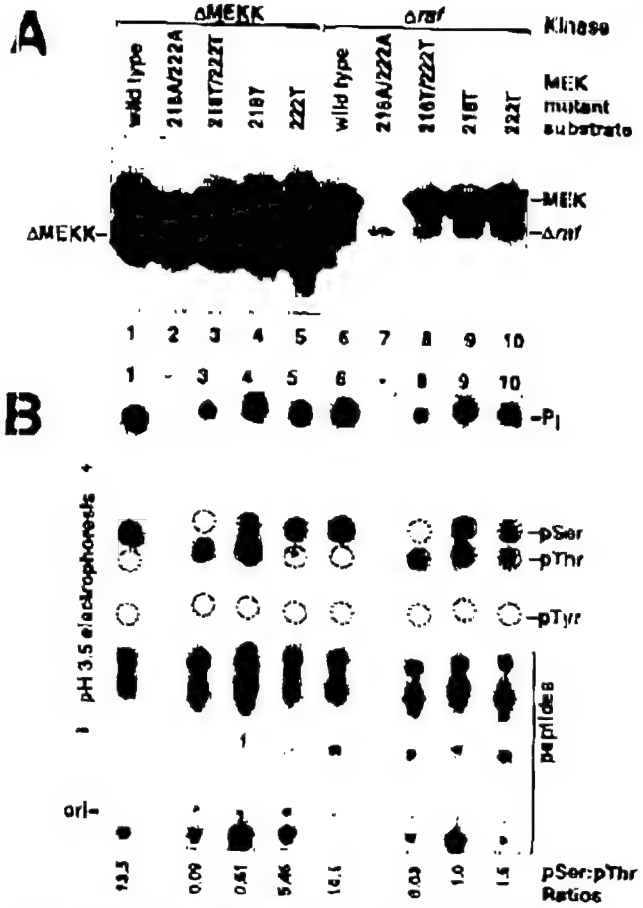

FIG. 3. **Identification of sites phosphorylated on MEK-1 by *raf* and MEKK.** Two MEK phosphorylation sites were identified as described in the text, and mutations of each site were made in which these sites were both altered to alanine residues or were changed singly or together to threonine residues. Each MEK protein also contained the inactivating K97R mutation to prevent autophosphorylation. *A*, phosphorylation of MEK mutants by *raf* or MEKK. Immunopurified MEK-1 or mutant proteins were incubated *in vitro* with immunopurified ΔMEKK (*lanes 1–5*) or with *Δraf* (*lanes 6–10*) together with ^{32}P-labeled ATP. The reaction products were separated by SDS-PAGE followed by transfer to Immobilon membranes for autoradiography. All mutant MEK proteins were phosphorylated to approximately the same level, with the exception of the S218A/S222A double mutant, which was not phosphorylated by either activating kinase. ΔMEKK was strongly autophosphorylated in this reaction, and *Δraf* was weakly autophosphorylated. *B*, phosphoamino acid analysis of *in vitro* phosphorylated MEK-1 mutants. Bands of radiolabeled MEK from the experiment shown in *A* (*lanes 1, 3–6,* and *8–10*) were excised from the Immobilon membrane and subjected to acid hydrolysis (see "Experimental Procedures"). Resultant hydrolysates were separated by one-dimensional thin layer electrophoresis at pH 3.5. The position of unlabeled phosphoamino acid markers is indicated by the *dotted circles*, as is that of free phosphate and the partially hydrolyzed peptides. Both ΔMEKK and *Δraf* are able to phosphorylate mutant threonine residues located at the position of the naturally phosphorylated serines. No serine phosphorylation is seen in the 218T/222T mutant, demonstrating that no other residues of MEK are phosphorylated by these kinases *in vitro*. Relative levels of phosphoserine and phosphothreonine quantified from this experiment are shown beneath each lane.

Mutation of the Activation Sites to Glutamic Acid Results in Constitutive Activation—Since phosphorylation of MEK-1 at codons 218 and 222 introduces negative charges into this portion of the protein and results in kinase activation, we conjectured that substitution of negatively charged amino acids at these two positions might similarly activate a mutant MEK-1 protein. Such a constitutively active allele would be predicted

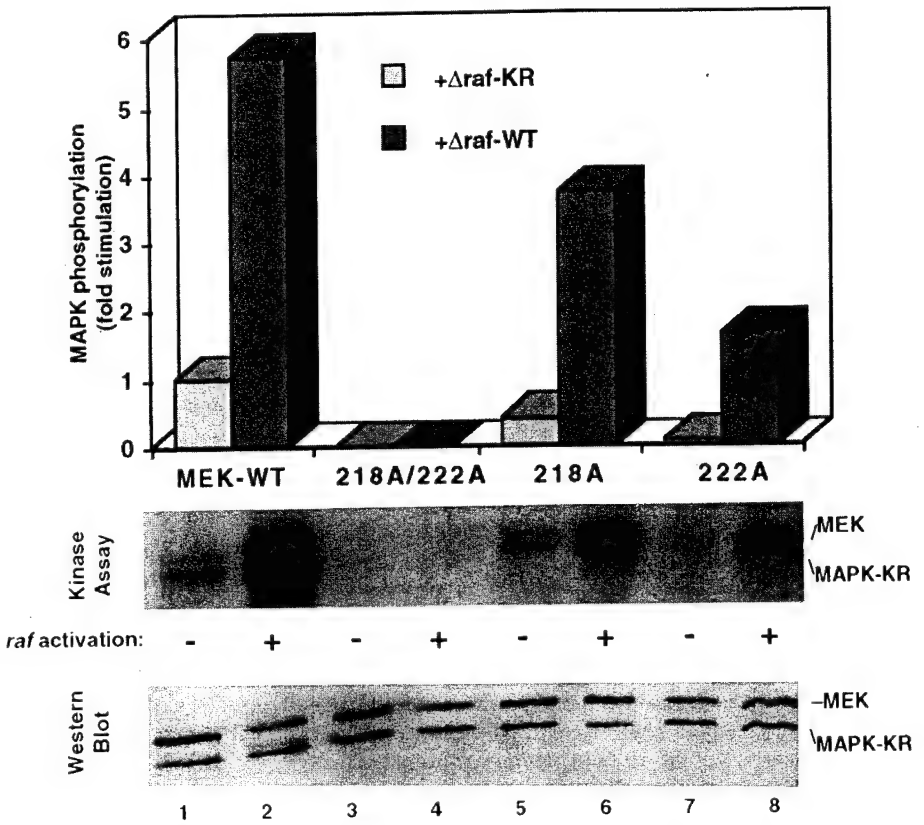

FIG. 4. **Ser[218] and Ser[222] are required for activation of MEK *in vivo*.** EE epitope-tagged MEK-WT, or alanine substitution mutants as indicated, were co-expressed with Δ*raf* or the inactive mutant Δ*raf*-K375R (that both lacked the EE epitope) to assess activation of MEK by Δ*raf*. EE-tagged MEK protein was specifically immunoprecipitated using anti-EE Affi-Gel beads and assayed for kinase activity by incubation with inactive epitope-tagged MAPK-KR substrate. Phosphorylated proteins were separated by SDS-PAGE and blotting onto Immobilon. Incorporation of radiophosphate into MAPK was quantified using an AMBIS β-imager and is shown in the *top panel*. The autoradiogram of this gel is shown in the *middle panel*. After quantification, the filter was probed using anti-EE mAb to verify equal recovery of kinases and substrate, shown in the *bottom panel*. Wild type MEK was active when expressed in the presence of *raf*, but not in the presence of the inactive mutant *raf*. Mutation of both sites to alanine codons completely prevented both basal MEK activity and *raf* activation. Mutation of individual serine codons to alanine resulted in partial activation. Autophosphorylation of MEK parallels phosphorylation of the MAPK substrate.

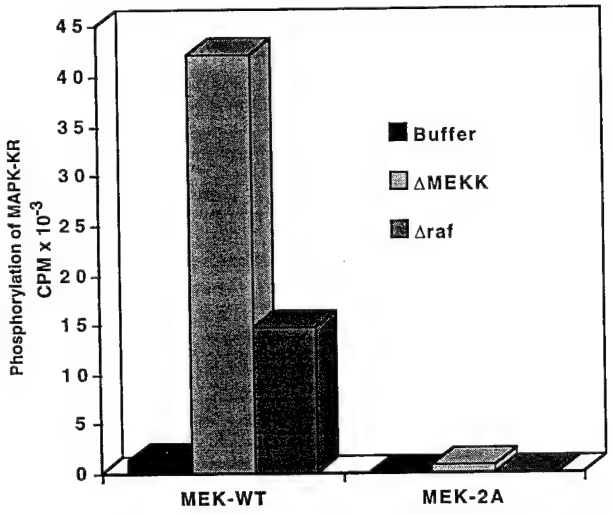

FIG. 5. **Ser[218] and Ser[222] are required for activation of MEK *in vitro* by MEKK or *raf*.** Immunopurified MEK-WT or MEK S218A/S222A mutant was activated *in vitro* with purified ΔMEKK, Δ*raf*, as described under "Experimental Procedures" using MAPK-KR as substrate. After SDS-PAGE and autoradiography, labeled MAPK was excised from the gel and quantified by Cérenkov counting. MEK-WT, but not the S218A/S222A, mutant was efficiently activated by ΔMEKK and Δ*raf*.

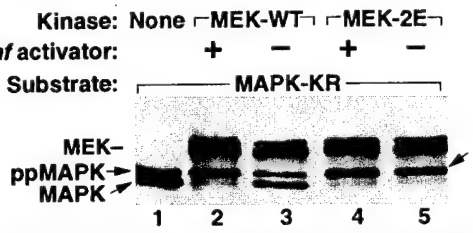

FIG. 6. **MEK-2E allele is constitutively active.** EE epitope-tagged MEK-WT or MEK-2E proteins were expressed together in cells with untagged Δ*raf* or the kinase-inactive Δ*raf*-KR mutant (designated + or − *raf* activator). Activity of the immunopurified MEK was detected using inactive MAPK-KR as substrate, detecting phosphorylation of MAPK by upward mobility shift on SDS-PAGE. MAPK is mostly unphosphorylated when incubated without MEK protein (*lane 1*). Incubation of the MAPK-KR substrate with Δ*raf*-activated MEK-1 results in nearly complete conversion of MAPK to the phosphorylated form (*lane 2*), but little conversion if MEK-1 is co-expressed with the inactive *raf* allele (*lane 3*). The MEK-2E mutant protein is also able to effect complete conversion of the MAPK-KR substrate to the phosphorylated form, either when expressed with (*lane 4*) or without (*lane 5*) active *raf* kinase. Thus MEK-2E activity is independent of upstream activation.

to be active independent of activation by phosphorylation by upstream kinases. To test this hypothesis, we constructed a mutant allele of MEK that contains the two mutations S218E and S222E, termed MEK-2E. Immunopurified MEK-2E was found to be catalytically active (see Fig. 6) but a more important question was whether the activity of the MEK-2E protein requires activation by upstream kinases.

In the experiment shown in Fig. 6, we expressed MEK-WT or MEK-2E together in cells with either Δ*raf* or the kinase-inactive Δ*raf*-KR mutant (neither *raf* allele was epitope tagged in this experiment). We then measured the ability of the immunopurified MEK to phosphorylate inactive MAPK-KR, detecting phosphorylation of MAPK by upward mobility shift on SDS-PAGE. Approximately 90% of immunopurified MAPK substrate is unphosphorylated (*lane 1*) when incubated without MEK protein. Incubation of the MAPK-KR substrate with Δ*raf*-activated MEK-1 results in nearly complete conversion of MAPK to the phosphorylated form (*lane 2*), but little conversion of MAPK is seen if MEK-1 is co-expressed with the inactive *raf* allele, demonstrating that activity of wild type MEK-1 protein is dependent upon activation by the co-expressed *raf* kinase. The MEK-2E mutant protein is also able to effect complete conversion of the MAPK-KR substrate to the phosphorylated form, but in contrast to the wild type MEK protein, MEK-2E is active when expressed either with or without active *raf* kinase

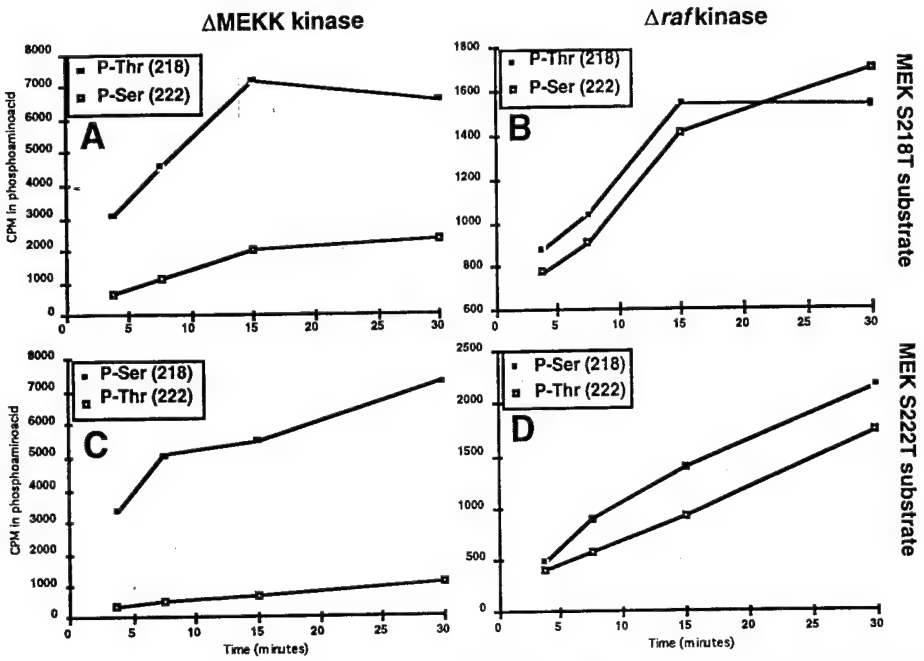

FIG. 7. **Time course of MEKK and *raf* phosphorylation of each activating residue.** To measure phosphorylation of MEK on codons 218 and 222, MEK S218T or S222T mutants were constructed, together with the inactivating K97R mutation of MEK-1. Each mutant MEK-1 protein was expressed separately and then immunopurified and reacted together with ΔMEKK or Δraf kinases. Parallel reactions were stopped after increasing time with SDS sample buffer and analyzed by SDS-PAGE. Radiolabeled MEK protein was subjected to phosphoamino acid analysis, and the amount of radiolabel associated with phosphoserine or phosphothreonine was quantified using an AMBIS β-detector. Four experiments are shown using either MEK S218T substrate (*A* and *B*) or MEK S222T (*C* and *D*) reacted with either ΔMEKK (*A* and *C*) or Δ*raf* (*B* and *D*) for the time shown. The amount of radiophosphoamino acid detected arising from each codon (either phosphoserine or phosphothreonine) is plotted separately. MEKK phosphorylates the residue at codon 218 more rapidly than the residue at codon 222 regardless of the hydroxy amino acid at that codon position. In contrast, Δ*raf* kinase phosphorylates both codons approximately equally at all time points.

(*lane 5*). Thus MEK-2E activity is independent of upstream activation.

MEKK and raf Have Distinct Specificity for Site Phosphorylation—The experiment shown in Fig. 3 suggests that phosphorylation of MEK-1 by *raf* results in approximately equal phosphorylation on serines at codons 218 and 222, whereas MEKK phosphorylates codon 218 preferentially. However, this distinction could reflect differences in the completion of the phosphorylation reaction rather than differences in the actual site preferences. Therefore we sought means of observing the kinetics of phosphorylation of each codon separately.

We first considered using synthetic peptide substrates to measure the site specificity of MEKK or *raf*. However, versions of MEK-1 containing large (50–100 codons) deletions distant to the activating sites have been completely unphosphorylated by MEKK or *raf*.[2] We interpret this to mean that an intact MEK protein is required for recognition of MEK-1 as a substrate by these kinases. It thus seems unlikely that peptide substrates could give meaningful results.

To measure phosphorylation of MEK on each of the two codons, we instead utilized MEK S218T and S222T mutations, together with the inactivating KR mutation. The reciprocal threonine mutations are important for measuring potential differences in *codon* specificity *versus* preference for serine over threonine residues. MEK S218T or S222T mutants were expressed separately and then immunopurified and reacted together with ΔMEKK or Δ*raf* kinases for increasing time periods. Radiolabeled MEK protein was subjected to phosphoamino acid analysis, and the amount of radiolabel associated with phosphoserine or phosphothreonine was quantified using an AMBIS β-detector.

Fig. 7 shows the analysis of the four experiments using the two substrates and the two kinases. MEKK was found to phosphorylate the residue at codon 218 more rapidly than the residue at codon 222 in reactions with both mutant MEK proteins (*A* and *C*). At later time points, phosphorylation on codon 218 plateaus and phosphorylation at codon 222 increases slightly. In contrast, Δ*raf* kinase (*B* and *D*) phosphorylated both codons approximately equally at all time points. When codon 222 was substitute with threonine, slightly less radioactive phosphothreonine was detected with either kinase. This may reflect that threonine is a slightly less preferred residue for both kinases. It is clear, however, that phosphorylation of MEK by MEKK and *raf* is biochemically distinguishable, although we have not detected biological consequences of this differentially phosphorylated MEK proteins.

DISCUSSION

Identification of the sites of activation of MEK-1 clarifies the role of MEKK and *raf* in transduction of cellular growth signals. The two sites of activating phosphorylation lie within a domain of MEK-1 between kinase domains VII and VIII (18). This activation domain of MEK-1 is conserved between MEK variants and species homologs. Fig. 8*A* shows the alignment of the comparable region of many MEK homologs across diverse species. In all cases, the 2 serines identified as MEK-1 activation sites are preserved, although in yeast and *Xenopus* the second serine is changed to a threonine residue.

Fig. 8*B* depicts the analogous regions of several serine-threonine protein kinases for which the sites of activating phosphorylation are known. In all examples found, activating phosphorylation also occurs between conserved kinase domains VII and VII. For the analogous activating region of MAPK, this region lies in a solvent-exposed portion of the protein, which has been termed the "activating lip" (34) and which may partially obstruct a substrate binding pocket. Spacial conservation of the

[2] M. Yan, unpublished observations.

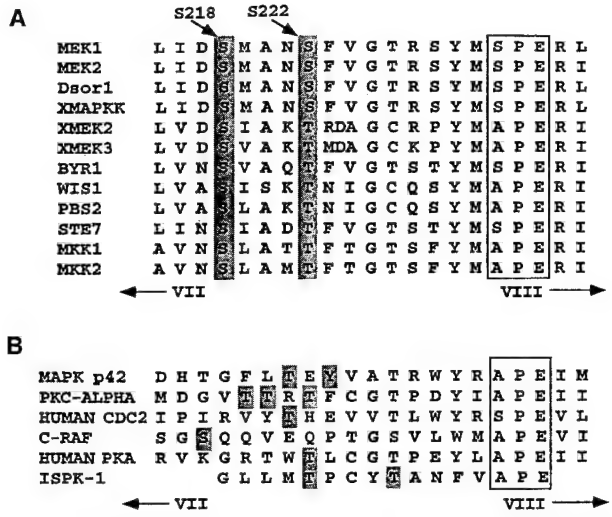

FIG. 8. **Comparison of the activation domain in homologs of MEK-1 and in other serine/threonine kinases.** *A*, amino acid sequences of MEK-1 and homologous proteins are compared in the region of the activating phosphorylation sites of MEK-1. *Shaded boxes* indicate serine or threonine residues conserved at the sites of MEK activation. Amino acid sequences of rat MEK-2 (MAP kinase kinase-2) (4) *Drosophila* Dsor-1 (19), *Xenopus* MAPKK (20), *Xenopus* MEK-2 and MEK-3 (21), *Schizosaccharomyces* byr-1 (22) and wis-1 (23), *Saccharomyces* PBS-2 (24), STE7 (25), and MKK1 and MKK2 (26) were from published sources. *B*, regions of several serine/threonine protein kinases between conserved domains VII and VIII are compared in which the kinases are known to be stimulated by phosphorylation in this region. *Shaded boxes* show sites of phosphorylation. Published sources identifying activating sites were used for MAPK (27), PKC-α (28), cdc2 (29, 30), c-*raf* (31), PKA (32), and ISPK-1 (33).

sites of activating phosphorylation suggests that this mode of regulation of kinase activity is strongly conserved, especially among kinases within signal transduction cascades. Activation sites in other kinases might thus be inferred by homology to this region.

The constitutive activity of our MEK-2E allele strongly supports the identification of codons 218 and 222 as sites of activating phosphorylation on MEK. Furthermore, this allele could prove to be a valuable reagent for analysis of signal transduction events, since it almost certainly contributes a continuous MEK-1 signal to the cell. We have recently constructed cell lines that express the MEK-2E allele in a regulated fashion and are beginning to measure the effects of this allele on growth signaling in cells. It should be noted that our strategy of substituting acidic residues in place of activating phosphorylation sites is not always successful. As a pertinent example, we have detected no activity in alleles of MAPK (obtained from Michael Weber) containing substitutions of glutamic acid for either the phosphorylated threonine, or tyrosine residues, or both.

Although MEK-1 is clearly activated by *raf* and MEKK, it is also capable of phosphorylating itself, as documented by several groups (20, 35). Using our S218T and S222T mutants we have confirmed that MEK-1 autophosphorylates both codons 218 and 222 and also phosphorylates uncharacterized threonine and tyrosine residues.

Our studies have shown clearly that phosphorylation of MEK-1 by MEKK and *raf* are not identical, with MEKK strongly preferring codon Ser^{218} as a site of phosphorylation. However, we do not know if this difference is reflected in biological differences in the signaling process effected by these two kinases. Using phosphorylation and activation of MAPK as a measure of MEK-1 function, both MEKK and *raf* seem equally able to activate MEK-1. The differences we observe in site preference might, however, reflect biological differences in signaling in several ways. For example, the two distinctly phosphorylated forms of MEK could recognize different substrates other than MAPK. Under this scenario doubly phosphorylated MEK (activated by *raf*) could phosphorylate an unknown substrate *critical for cell transformation* that is not recognized by MEKK activated MEK-1. Alternatively, one of the differently phosphorylated MEK-1 forms could remain activated longer within the cell. Since phosphatase(s) that inactivate MEK-1 have not been characterized, it is possible that a separate phosphatase is responsible for dephosphorylating each residues. If this were true, termination of the two activating events might be separately regulated. Since our data indicate that singly phosphorylated MEK-1 remains partially activated (see Fig. 4), a phosphatase specific for codon 218 could specifically negate signals arising from MEKK activation while leaving the signal arising from *raf* partially intact.

Irrespective of mechanism, the differences between MEK-1 signal transduction effected by MEKK or *raf* is significant for one overriding reason: *raf* has clearly been identified as a component of an oncogenic kinase cascade, whereas MEKK has not. Alternative phosphorylation of MEK-1 protein, resulting in similar yet distinct activation of MEK-1, could be a means by which oncogenic *versus* non-oncogenic growth signals are propagated.

Since this work was completed, a report identifying MEK codons 218 and 222 as substrates for *raf* has been published (36).

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Appendex B Yan, 1

Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1

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A KINASE distinct from the MEK activator Raf[1–3], termed MEK kinase-1 (MEKK), was originally identified by virtue of its homology to kinases involved in yeast mating signal cascades[4]. Like Raf, MEKK is capable of activating MEK *in vitro*[4,5]. High-level expression of MEKK in COS-7 cells[4] or using vaccinia virus vectors[5] also activates MEK and MAPK, indicating that MEKK and Raf provide alternative means of activating the MAPK signalling pathway. We have derived NIH3T3 cell sublines that can be induced to express active MEKK. Here we show that induction of MEKK does not result in the activation of MAPK, but instead stimulates the stress-activated protein kinases (SAPKs)[6–8] which are identical to a Jun amino-terminal kinase[9,10]. We find that MEKK regulates a new signalling cascade by phosphorylating an SAPK activator, SEK1 which in turn phosphorylates and activates SAPK.

Stably transfected NIH3T3 subclones express MEKK in response to isopropyl-β-D-thiogalactosidase (IPTG) (Fig. 1*a*) but MAPK activity remains unchanged (Fig. 1*b*). In contrast, SAPK activity is increased six- to eightfold in MEKK-inducible cell lines but not in the parent NIH3T3 cells. These MEKK-expressing cells are able to activate MAPK in response to some mitogenic signals, because treatment with phorbol ester increases MAPK activity in each of these clones (as well as in NIH3T3 cells), whereas SAPK activity is unaffected (Fig. 1*c*). Increased SAPK activity is evident by 3 hours and maximal after 12 hours of induction; MAPK activity is unchanged throughout the 23-hour incubation with inducer (Fig. 1*d*). Together these results indicate that, in contrast to the presumed role of MEKK in activating MEK and MAPK, MEKK acts instead to activate SAPKs. Expression of truncated ΔMEKK in these clones resulted in six- to eightfold inhibition of growth rate compared with parental NIH3T3 cells.

We modelled activation of SAPK by MEKK using cloned genes and purified proteins expressed using vaccinia virus vectors[11,12]. MEKK induced electrophoretic retardation of SAPK, which was suggestive of quantitative phosphorylation (Fig. 2*a*), and also increased the amount of phosphotyrosine in SAPK and activated its Jun N-terminal kinase activity. Thus, in this overexpression model as well as in the inducible cell line, MEKK expression results in activation of the SAPK pathway.

We considered the possibility that activation of SAPK occurred as a consequence of activation of the MEK and MAPK cascade. To stimulate MAPK independently of MEKK, we used activated Raf and a constitutively active allele of MEK1 termed MEK 2E (ref. 5). Both Raf and MEK 2E were able to induce phosphorylation of coexpressed MAPK (Fig. 2*b*). Neither of these MAPK activators induced phosphorylation of SAPK, indicating that the SAPK activation pathway is effectively insulated from the MAPK pathway.

MEKK was unable to phosphorylate SAPK *in vitro* (below). We therefore tested whether MEKK activated the newly identified SAPK activator, SEK1 (ref. 13), whose sequence is similar to MEK1. Immunopurified MEKK (but not the inactive mutant

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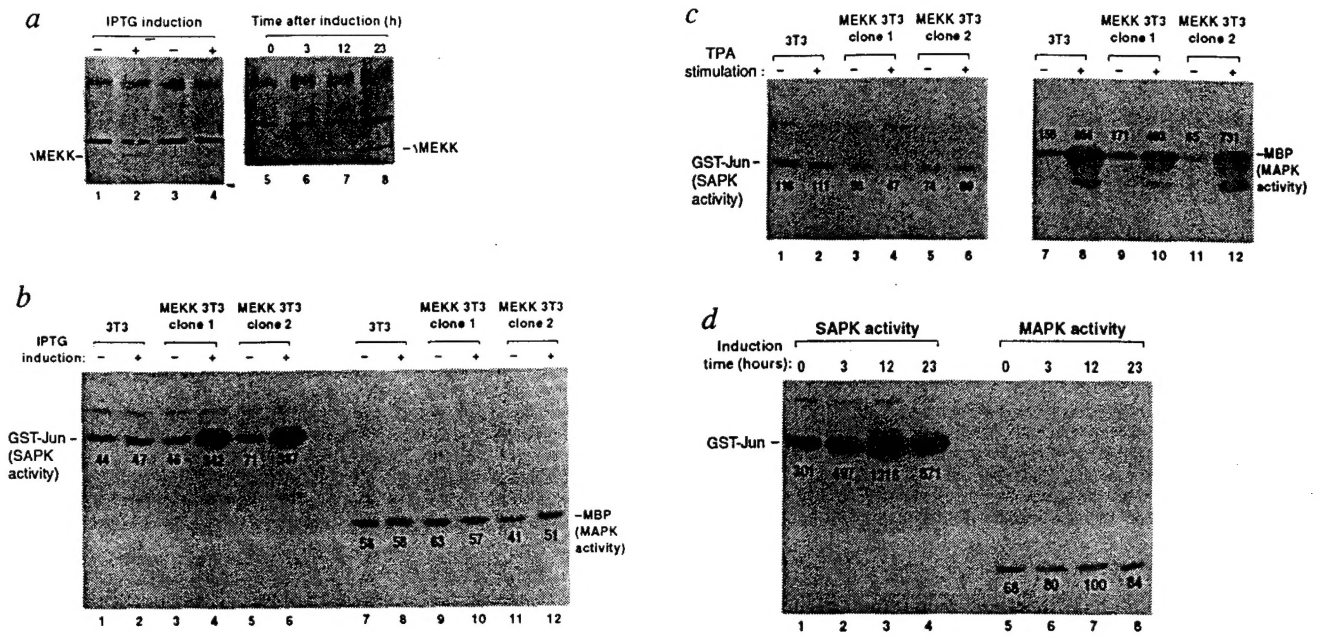

FIG. 1 *a*, MEKK expression in NIH3T3 cells. Epitope-tagged truncated MEKK (ΔMEKK) was detected in MEKK clone 1 cells (lanes 1 and 2) or in MEKK clone 2 cells (lanes 5–8) but not in a control clone (lanes 3 and 4) treated with IPTG for 24 h (lanes 2 and 4) or at the indicated times (lanes 6–8). *b*, MAPK and SAPK activity in MEKK-inducible cell lines. Numbers below labelled bands indicate c.p.m. of radioactivity in substrates. SAPK activity, but not MAPK activity, was increased in response to MEKK expression. *c*, Functional MAPK signalling in NIH3T3 cells and MEKK-expressing subclones after stimulation (+) with 250 ng ml^{-1} TPA. *d*, Time course of induction of SAPK activity in MEKK 3T3 clone 2 cells after IPTG treatment. MAPK activity throughout this period remained unchanged, whereas SAPK activity was increased even at the 3-h time point, when MEKK expression could not yet be detected. MEKK expression in cells used here is shown in *a*, lanes 5–8.

METHODS. The EE epitope-tagged[5] C-terminal 320 amino acids of MEKK1 (ΔMEKK) was expressed in NIH3T3 cells using the lacSwitch promoter (Stratagene). ΔMEKK was induced in cell clones with 1 mM IPTG and detected by immunoprecipitation and immunoblotting using the anti-EE monoclonal antibody (mAb). MAPK and SAPK activity was determined using polyclonal antibodies recognizing a C-terminal peptide of p42MAPK or a p54SAPK–GST fusion protein. Immune complexes containing protein from 10^5 cells were reacted with 0.5 μg GST–Jun (amino acids 5–89; ref. 8) for SAPK, or MBP (Sigma) for MAPK in 20-μl reactions (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 15 μM ATP, 5 μCi [^{32}P-γ]ATP), for 30 min at room temperature. Radioactivity was quantified using an AMBIS β-detector.

MEKK(K → R) rapidly phosphorylated a glutathione-*S*-transferase(GST)–SEK fusion protein on serine and threonine residues (Fig. 3*a*) but failed to phosphorylate a SEK mutant in which the two residues equivalent to the sites of activation in MEK were mutated. Phosphorylation of GST–SEK activated the SAPK activity of GST–SEK. Thus, SEK is a substrate of MEKK and phosphorylation by MEKK is sufficient to activate SEK. MEKK expression also activates SEK *in vivo* (Fig. 3*b*). Activation of SAPK by MEKK requires functional SEK because coexpression of a dominant inhibitory allele of SEK blocks activation of SAPK by MEKK (Fig. 3*c*).

Coexpression of full-length MEKK protein is able to effect phosphorylation of SEK and activation of SAPK (Fig. 4*a*), similar to the activation induced by truncated MEKK. The high activity of full-length MEKK protein during overexpression suggests that a cellular activity might regulate natural MEKK expressed at lower levels. This result is in contrast to Raf, which displays low levels of kinase activity unless truncated[14].

Our results demonstrate complete reconstitution of a kinase cascade, beginning with MEKK, that phosphorylates and activates SEK, which subsequently phosphorylates and activates SAPK. Each component of this cascade is functionally parallel

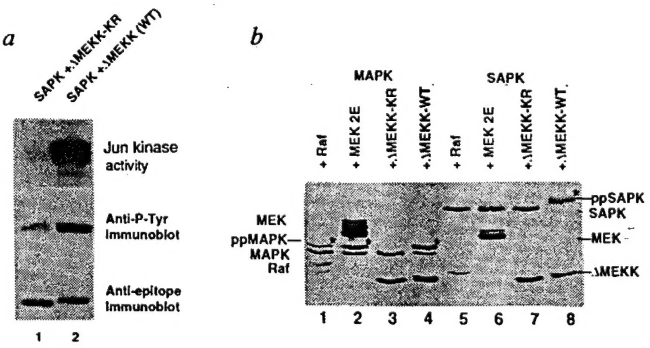

FIG. 2 Activation of SEK–SAPK pathway by coexpression of ΔMEKK using vaccinia virus vectors. *a*, Epitope-tagged SAPK was expressed with either the untagged inactive K → R mutant of ΔMEKK (lane 1) or wild-type ΔMEKK (lane 2). Coexpression of active MEKK resulted in mobility shift of SAPK detected by anti-epitope immunoblotting (bottom panel) and also increased tyrosine phosphorylation of SAPK detected in anti-epitope immunoprecipitates (middle panel). SAPK activity was also strongly elevated, reflected by phosphorylation of GST–Jun(5–89) using anti-epitope immunoprecipitates (top panel). *b*, epitope-tagged MAPK (lanes 1–4) or SAPK (lanes 5–8) was expressed with epitope-tagged forms of truncated active Raf (lanes 1, 5), constitutively active MEK 2E (lanes 2, 6), ΔMEKK(K → R) mutant (lanes 3, 7) or ΔMEKK wild type (lanes 4, 8), and detected in whole cell lysates using anti-epitope western blot. Activation of both MAPK and SAPK is identifiable by the appearance of bands with delayed mobility, indicated by stars. pp prefix, phosphorylated protein forms. Raf and active MEK 2E are able to activate MAPK, but not SAPK, thus the SAPK pathway is insulated from the MAPK pathway. MEKK is able to activate MAPK in this overexpression system, though it is not when expressed at lower levels (Fig. 1). Of the kinases tested, only MEKK is able to activate SAPK.

METHODS. The N-terminal EE-epitope-tagged p54SAPKα1, and untagged ΔMEKK were expressed using the vaccinia virus expression system and the plasmid pTM1 (ref. 12). Phosphotyrosine was detected using mAb 4G10 (UBI) to probe anti-EE immunoprecipitates. Jun kinase was assayed as for Fig. 1. Kinase expression and blotting has been described[5].

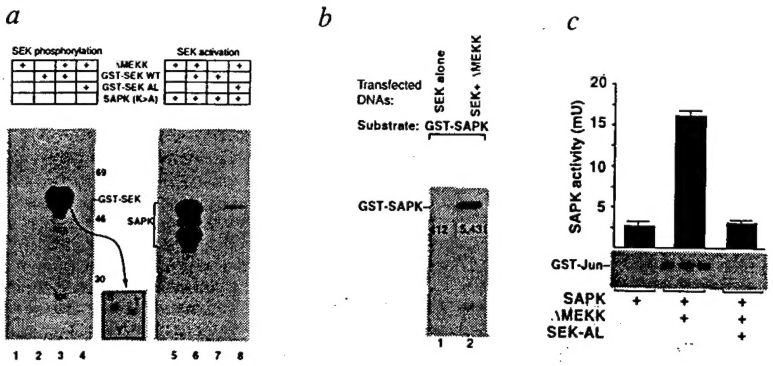

FIG. 3 *a*, MEKK phosphorylates and activates of SEK *in vitro*. Immunopurified ΔMEKK phosphorylated wild-type GST–SEK (lane 3) on serine and threonine (see phosphoaminoacid analysis, inset), but not mutant GST–SEK protein lacking the two phosphorylation sites (lane 4). GST–SEK1 phosphorylated by MEKK *in vitro* acquired SAPK kinase activity, as shown after secondary reaction with inactive (K>A) mutant thrombin-cleaved GST–SAPK with radioactive ATP (lane 6). Mutant GST–SEK protein lacking phosphorylation sites (lane 8) or reactions without either MEKK or SEK did not allow phosphorylation of SAPK. *b*, MEKK activates SEK1 *in vivo*. Epitope-tagged SEK expressed in CV1 cells using a CMV expression vector (lane 1) became activated by coexpression of ΔMEKK lacking the epitope tag (lane 2). Anti-epitope immunocomplexes were assayed for SEK activity using GST–SAPK as substrate (see Fig. 1 legend). *c*, SAPK activation by MEKK requires SEK1. Epitope-tagged SAPK expressed using SV40-based vectors was activated by coexpression with ΔMEKK. This activation was reversed by triple coexpression of a dominant inhibitory mutant of SEK1 containing (S220A, T224L; SEK AL).

METHODS. Epitope-tagged ΔMEKK was expressed, immunopurified and eluted using excess EE peptide[5]. Bacterial SEK–GST fusion protein was purified and reacted *in situ* on glutathione–agarose beads. Activated MEKK was separated from GST–SEK by washing the glutathione beads, and subsequently incubated with SAPK substrate (cleaved from GST by thrombin, and containing an inactivating K55A mutation) in kinase reactions containing [^{32}P]ATP (20 mM ATP total concentration). Inhibition of HA-epitope-tagged SAPK by SEK-S220A, T224L (SEK-AL) was tested in L929 cells using the SV40-based pMT2 vector; 7 μg SAPK, 7 μg ΔMEKK and 15 μg SEK-AL expression plasmids were transfected together with empty pMT2 vector DNA to equalize plasmid mass. SAPK assays represent triplicate measurements using GST–Jun substrate as described[13].

to a component of the MAPK activation pathway: SAPK is analogous to MAPK, SEK analogous to MEK, and MEKK analogous to Raf (Fig. 4*b*). Other evidence suggests that SAPK signalling in response to ultraviolet irradiation[10,15] and tumour-necrosis factor-α[16] lies downstream of Ras. Additionally, dominant inhibitory Ras reduces the activity of MEKK[17] and the MEKK homologue Byr2 associates with Ras1 in yeast[18]. Thus, in parallel to Raf–MEK–MAPK, the MEKK–SEK–SAPK pathway most probably lies downstream of Ras. Cofactors for Ras must exist that contribute specifically to either the mitogenic or stress-response pathways.

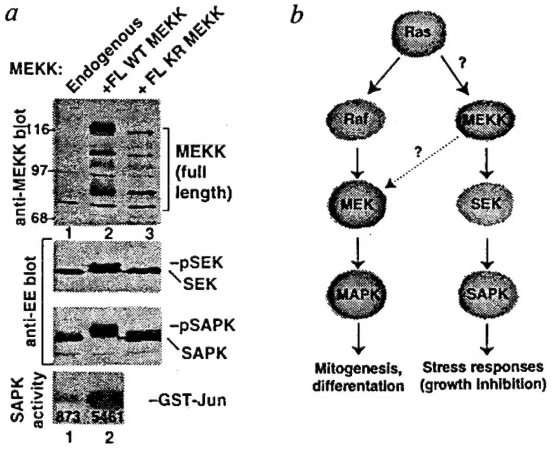

FIG. 4 *a*, Activation of SEK and SAPK *in vivo* by full-length MEKK. Using the vaccinia virus expression system in CV1 cells, SEK1 or SAPK were separately expressed alone (lane 1) or with vectors encoding full-length wild-type MEKK1 (lane 2) or a kinase-inactive (K447R) mutant allele of MEKK1 (lane 3). Expression of several bands related to full-length MEKK (top panel) was detected using chicken polyclonal antibodies raised against bacterially expressed ΔMEKK. Endogenous proteins of 70K, 90K and 110K were also detected (lane 1). Epitope-tagged SEK1 and SAPK1 (middle panels) were detected by immunoblotting. Electrophoretically retarded bands arising from phosphorylation of both SEK and SAPK were observed from coexpression with full-length MEKK, indicating that MEKK thus expressed is constitutively active. SAPK activity was activated by full-length MEKK (bottom panel). *b*, Diagram of separate pathways emanating from Raf and MEKK. Both Raf and MEKK appear to be dependent upon the function of Ras, as described in the text. The Raf–MEK–MAPK pathway is functionally analogous to the MEKK–SEK–SAPK pathway, although the result of stimulation of each pathway is distinct. A dotted arrow from MEKK to MEK reflects the ability of MEKK to phosphorylate MEK *in vitro* and during high-level cell expression. In stable inducible cell lines, MAPK activation by MEKK is not seen, drawing the physiological significance of this path into question. The opposing nature of two signalling pathways both emanating from Ras suggests an important role for factors cooperating with Ras to provide specificity for stimulation of one path versus the other.

Is MEKK able to activate MEK, as originally proposed? When tested *in vitro* or during overexpression[4,5], MEKK is able to phosphorylate and activate MEK. But with the stable MEKK-inducible NIH3T3 cells studied here, MEKK, through SEK, activates SAPK and not MAPK, even though these cells express an intact MAPK activation pathway. MEKK might stimulate MAPK in other cell types, or transiently, although we detected no MAPK activity as early as 3 hours, at which time SAPK activity was raised and MEKK was undetectable. Additionally, activated *Drosophila* MEK (Dsor1) rescues D-Raf null mutants in both the Torso and R7 photoreceptor pathways[19], implicating Raf as the major physiological MEK activator.

Perhaps it is appropriate to consider MEKK versus RAF signalling as analogue rather than binary cell regulation. Depending on the interplay between kinase activities, substrate availability and the intracellular milieu, MEKK might activate SAPK in our experimental systems; other cellular conditions might translate MEKK activity into a variety of mixed signals involving other homologues of MAPK[20], including SAPK. □

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